Cloning of Pectate Lyase Gene *pel* from *Pseudomonas fluorescens* and Detection of Sequences Homologous to *pel* in *Pseudomonas viridiflava* and *Pseudomonas putida*

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Pectate lyase (PL) depolymerizes pectin and other polygalacturonates (PGAs) and is thought to play a role in bacterial invasion of plants. Production of PL by the soft-rotting pathogen *Pseudomonas fluorescens* CY091 is regulated by Ca^{2+}. In the presence of Ca^{2+}, this bacterium constitutively synthesizes PL in media containing glucose, glycerol, or PGA and excretes over 87% of total PL into culture fluids. In the absence of Ca^{2+}, the organism fails to use PGA as a carbon source and produces very low levels of PL in media containing glucose or glycerol. Of the small amount of PL produced by the bacterium in Ca^{2+}-deficient media, over 78% was detected within the cells, indicating that Ca^{2+} is critical not only for the production but also for the secretion of PL. The *pel* gene, encoding an alkaline PL (pI 10.0, M_r 41,000) was cloned and located on the overlapping region of a 4.3-kb *Sal*I and a 7.1-kb *Eco*RI fragment. The 7.1-kb *Eco*RI fragment appears to contain a promoter for *pel* gene expression. A 1.7-kb *Sal*I-*Xho*I subfragment of the 4.3-kb *Sal*I fragment was cloned into pUC18 to give pROTM2. *Escherichia coli* cells carrying pROTM2 produce 50 to 100 times more PL than do cells carrying other pectolytic constructs. Production of PL by *E. coli*(pROTM2) was not affected by carbon sources or by Ca^{2+}. The pI and M_r of PL from *E. coli* corresponded to values for its counterpart from *P. fluorescens*. A 0.7-kb *Bgl*II-*Cla*I fragment encoding the *pel* structural sequence was used to detect *pel* homologs in various species of fluorescent pseudomonads. Homologous sequences were observed in 10 of 11 strains of *P. fluorescens*, *P. viridiflava*, and *P. putida*. The *pel* gene in fluorescent pseudomonads is well conserved and may exist and remain repressed in certain strains or species which exhibit nonpectolytic phenotypes under laboratory conditions.

Pseudomonas fluorescens is a heterogenous species and consists of a diversity of ecologic and physiological groups (36). Certain strains of this species are postharvest pathogens of plants, which cause soft rot of fruits and vegetables in storage and at markets (25). Soft-rotting *P. fluorescens* (often referred to as *P. marginalis*) is capable of degrading pectic components of plant cell walls by producing a wide variety of pectolytic enzymes, including pectin methylesterase (30), pectin lyase (33, 35), polygalacturonase (10, 30, 42), and pectate lyase (PL) (9, 10, 12, 22, 30, 42). Production of methylesterase, pectin lyase, and polygalacturonase is rare and has been detected only in a few strains (30, 33, 35, 42). With the exception of one strain (30), all of the soft-rotting pseudomonads so far studied produce PL. Thus, it is generally believed that PL is the principal or sole enzyme responsible for tissue maceration caused by most strains of *P. fluorescens* and *P. viridiflava* (22, 24).

The PL system of *P. fluorescens* is considerably different from that of *Erwinia* spp., although soft-rot symptoms caused by the two organisms are similar. In *Erwinia* spp., all strains so far studied produce three to five PL isozymes (pI 4.5 to 10.0) (18), whereas in *P. fluorescens*, all eight strains recently examined in our laboratory produce one or possibly two PLs (22). Furthermore, production of PLs in *Erwinia* spp. is induced by pectic substrates and subjected to catabolite or self-catabolite repression (18). In *P. fluorescens*, however, the mode of PL production varies considerably among strains and can be constitutive (30, 42) or inducible (9, 30, 43). In some strains, PL production is induced by pectic substances in a mechanism resembling that previously demonstrated in *Erwinia* spp. (18). In others, the regulatory factors or mechanisms have not been clearly defined. For example, Zucker and Hankin (43) showed that a nonpectolytic isolate of *P. fluorescens* can be converted to pectolytic by a series of subcultures in media containing pectin or plant tissue extracts. Hildebrand (10) reported that expression of a pectolytic phenotype in fluorescent pseudomonads is greatly influenced by various factors in culture media. Also, it has been observed in our laboratory that certain isolates of *P. fluorescens* tend to lose pectolytic and pathogenic phenotype after several passages in vitro (23a). A similar phenomenon has been reported in strains of *P. viridiflava* (3, 24). All of these observations indicate that genetic and biochemical mechanisms regulating PL production in fluorescent pseudomonads are unique. The simplicity of the PL system as revealed in *P. viridiflava* (24) and *P. fluorescens* (22) makes either organism a useful model for the investigation of the synthesis and secretion of extracellular proteins by prokaryotes.

The genes (*pel*) encoding PL in diverse groups of microorganisms, including *Erwinia* spp. (18), *Xanthomonas campestris* pv. campestris (8), *Yersinia pseudotuberculosis* (28), and *Aspergillus nidulans* (7), have recently been cloned and characterized. Despite its pathologic and ecologic importance, the *pel* gene of fluorescent pseudomonads has not yet been carefully examined. Molecular cloning of the *pel* gene from *P. fluorescens* would enable the study of the structure and organization of this gene and examination of the mechanisms that regulate synthesis and secretion of PL. In addition, the cloned gene could be used as a probe to assess the pectolytic and pathogenic potential of strains that show a nonpectolytic phenotype under conventional testing

TABLE 1. Bacterial strains

Strain	Biovar[a]	Pectolytic phenotype[b]	Isolated from:	Source or reference[c]
P. fluorescens				
CY091	II	+	Celery	25
17816	II	+	Dahlia	ATCC
PJ-08-30	II	+	Pepper	25
SJ-0802	II	+	Squash	25
BC-05-1B	V	+	Broccoli	25
LC-04-2B	V	+	Lettuce	25
AJ-06-2A	V	+	Asparagus	25
13525	I	−	Water	ATCC
P. viridiflava SF-312		+	Squash	24
P. putida				
AG8	A	−	Pepper	23
PP22	B	−	Pepper	23
E. coli HB101				BRL

[a] Determined as previously described (36).
[b] Pectolytic and tissue-macerating abilities were assayed as described in the text.
[c] ATCC, American Type Culture Collection; BRL, Bethesda Research Laboratories.

TABLE 2. Plasmids

Plasmids	Description[a]	Source or reference
pBR322	Cloning plasmid	1, BRL[b]
pBR325	Cloning plasmid	1, BRL
pUC18	Cloning plasmid	41, BRL
pROT1	pel^+ clone of strain CY091	This study
pROT2A	7.1-kb *Eco*RI fragment from pROT1 cloned in pBR325, pel^+	This study
pROT3A	Same construction as pROT2A except cloned in the opposite orientation of the vector Cm^r promoter, pel^+	This study
pROT3A2	5.3-kb *Eco*RI-*Bam*HI fragment from pROT3A cloned in pBR322, pel^+	This study
pROTX1-X3	*Eco*RI and *Bam*HI subclones of pROT3A, pel	This study
pROT8B	4.3-kb fragment from pROT1 cloned in pBR322, pel^+	This study
pROTM2	1.7-kb *Sal*I-*Xho*I fragment from pROT8B cloned into the *Sal* site of pUC18 in the orientation of the *Xho*I site downstream of the vector *lac* promoter, pel^+	This study
pROTM21	Same construction as pROTM2 except cloned in the opposite orientation, *pel*	This study

[a] Cm^r, chloramphenicol resistance; pel^+, pectolytic; and *pel*, nonpectolytic.
[b] BRL, Bethesda Research Laboratories.

conditions. This information is becoming more important since a great number of *P. fluorescens* and *P. putida* strains are being considered for introduction into environments for improvement of plant growth or for control of plant pests (23). At present, it is not known for sure that all of these pseudomonads are nonpathogens and do not cause deleterious effects on plants. More knowledge about genetic and biochemical mechanisms governing PL production in fluorescent pseudomonads is needed. This information will provide a basis for the development of new control strategies or the production of beneficial pseudomonads that are environmentally safe for agricultural applications.

The objectives of this study were to (i) define some of the conditions that affect PL production by *P. fluorescens*, (ii) clone and characterize the *pel* gene coding for PL in this organism, and (iii) determine the occurrence and genomic organization of *pel*-homologous sequences in various species of fluorescent pseudomonads, including *P. fluorescens*, *P. putida*, and *P. viridiflava*.

(Preliminary results of the study have been presented at the Annual Meeting of the American Society for Microbiology, New Orleans, La., 14 to 18 May 1989, and at the Fifth Fallen Leaf Lake Conference on Molecular Biology of Bacterial Plant Pathogens, South Lake Tahoe, Calif., 14 to 17 September 1989.)

MATERIALS AND METHODS

Bacterial strains and plasmids. *Pseudomonas* spp. and plasmids used in the study are described in Tables 1 and 2. *P. fluorescens* CY091 was originally isolated from a rotted specimen of celery (25). This strain causes maceration of celery tissues more effectively than do strains isolated from other plants (23a). Additionally, this strain possesses biochemical and nutritional properties that fit the typical phenotype of *P. marginalis* as described by Lelliott et al. (21) and the typical phenotype of biotype B (or biovar II) of *P. fluorescens* as described by Stanier et al. (36). *Escherichia coli* HB101 and cloning vectors (pBR322 [1], pBR325 [1], and pUC18 [41]) were obtained from Bethesda Research Laboratories (Gaithersburg, Md.).

Media and culture conditions. Luria broth (LB; GIBCO Laboratories, Grand Island, N.Y.) was used for cultivation of both *E. coli* and *Pseudomonas* spp. When a solid medium was required, *E. coli* and *Pseudomonas* spp. were grown on Luria agar (LA) and on *Pseudomonas* agar F (Difco Laboratories, Detroit, Mich.), respectively. Minimal salt (MS) solution contained K_2HPO_4 (0.7%), KH_2PO_4 (0.2%), $MgSO_4 \cdot 7H_2O$ (0.02%), and $(NH_4)_2SO_4$ (0.1%) at pH 7.1. As needed, $CaCl_2$ was added to MS solution to a final concentration of 1 mM. Three carbohydrates (glucose, glycerol, and PGA) were examined as carbon sources and were used at final concentrations of 0.2%, 0.2%, and 0.4%, respectively. For *E. coli* clones, MS medium was further enriched with yeast extract (0.1%) and Casamino Acids (0.3%; Difco). If required, antibiotics were added at the following concentrations: ampicillin, 50 $\mu g\ ml^{-1}$; tetracycline, 12.5 $\mu g\ ml^{-1}$; and chloramphenicol, 20 $\mu g\ ml^{-1}$. Unless otherwise indicated, *E. coli* and *Pseudomonas* spp. were cultured at 37 and 28°C, respectively.

Assay of PL activity. A semisolid pectate (SSP) medium (pH 7.1 ± 1) containing sodium polypectate (1.8%; Sunkist Growers, Inc., Ontario, Calif.), yeast extract (0.2%), $CaCl_2$ (5 mM), and agar (0.1%) was prepared as previously described (37). This medium was routinely used to assay for pectolytic activity of *Pseudomonas* isolates and to screen for *E. coli* clones carrying the *pel* gene. Positive isolates were identified by the formation of a pit underlying and surrounding the bacterial growth. To quantitate PL activity, a spectrophotometric method which measures the A_{232} of catalytic end products (unsaturated uronides) was used (24). Reactions were carried out at 30°C in 0.5-ml mixtures containing 100 mM Tris · HCl (pH 8.0), $CaCl_2$ (1 mM), PGA (0.2%; Sigma Chemical Co., St. Louis, Mo.), and enzyme sample. One unit of activity is defined as the amount of enzyme which causes an increase of 1.73 absorbance units per min

(42). The protein concentration was determined by the method of Bradford (4).

Assay of tissue maceration. A modification of the method of Maher and Kelman (26) was used. Bacteria were grown in LB, and cell pellets obtained by centrifugation were washed and resuspended in 0.1 M sodium phosphate-buffered saline (pH 7.0) to a final cell concentration of 10^7 CFU ml^{-1}. Potato tubers (Russet Burbank) were surface sterilized in 1% NaOCl for 10 min, rinsed three times with sterile water, and dried. A 100-μl pipette tip was used to make holes to 20 mm in depth in intact tubers. Ten microliters of bacterial suspension was injected into each hole. The inoculated hole was sealed with Vaseline, and tubers were wrapped with Saran Wrap (Dow Chemical Co.). Maceration of potato tissue was visually examined after 7 days of incubation in a moist chamber at 20°C.

Effect of carbon sources and Ca^{2+} on PL production. MS media containing glucose, glycerol, or PGA and with or without $CaCl_2$ were prepared. Each medium was inoculated with stationary-phase cells of *P. fluorescens* CY091 to give an initial cell density of approximately 3×10^5 CFU ml^{-1}. Cultures were incubated at 28°C with shaking (125 rpm) for 50 h. Cells were then separated from the culture medium by centrifugation (10,000 × *g*, 10 min), and the supernatant was removed and assayed for extracellular PL activity. The cell pellet was washed once in 50 mM Tris · HCl (pH 8.0) and resuspended in the same buffer at 1/10 volume of the original culture medium. Cells were subsequently disrupted by ultrasonication (24), and cell debris was removed by centrifugation (25,000 × *g*, 30 min). The clear supernatant thus obtained was used to determine the intracellular or cell-bound PL activity.

Cloning, subcloning, and restriction mapping. *P. fluorescens* CY091 chromosomal DNA was isolated by the method of Shepard and Polisky (34). DNA was partially digested with *Sau*3A and fractionated by sucrose gradient centrifugation (27). Fractions containing 12- to 18-kb fragments were pooled, dialyzed, and further purified on an Elutip-d minicolumn (Schleicher & Schuell, Keene, N.H.). pBR322 DNA was digested with *Bam*HI and dephosphorylated with calf intestine alkaline phosphatase. Ligation of pBR322 DNA and *P. fluorescens* DNA with T4 DNA ligase was carried out at 14°C for 18 h. Competent cells of *E. coli* HB101 prepared by the $CaCl_2$ procedure were transformed with ligated DNA samples as previously described (27). Transformants that appeared on LA-ampicillin plates were screened for pectolytic activity on SSP medium as described above.

Restriction mapping and subcloning were done by standard procedures (27). Deletion derivatives were constructed by digesting the parent plasmid with one or two endonucleases and ligating the resulting products with T4 DNA ligase. For subcloning, desired DNA fragments were isolated from agarose gels by electroelution (27) or from low-melting-point agarose gels following electrophoresis (6). Specific DNA fragments were ligated with pBR322, pBR325, or pUC18 DNA previously digested with appropriate restriction endonucleases. Subclones were assayed for pectolytic activity, and insertion fragments were further analyzed by restriction endonucleases. Enzymes used in the cloning experiments were obtained from Bethesda Research Laboratories or Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Plasmids were analyzed by the rapid procedures of Kado and Liu (14) and purified by centrifugation in cesium chloride gradients (27).

Localization of PL in *E. coli* cells. Pectolytic *E. coli* clones were grown in MS media enriched with 0.1% yeast extract and 0.3% Casamino Acids. The medium was inoculated with stationary-phase cells to give an initial concentration of approximately 3×10^5 CFU ml^{-1} and incubated with shaking at 28°C for 16 h. Cells were separated from culture medium by centrifugation (10,000 × *g*, 10 min), and the supernatant was used to assay for extracellular PL activity. Cell pellets were washed once and resuspended in 0.2 M Tris · HCl (pH 8.0). Osmotic shock fluids were prepared by the method of Witholt et al. (40). Spheroplasts were first separated by centrifugation (12,000 × *g*, 20 min), and periplasmic fluid retained in the supernatant was collected and used to determine periplasmic PL activity. Next, spheroplasts were disrupted by ultrasonication, and the sonic extract was centrifuged (20,000 × *g*, 20 min). The clear supernatant was then assayed for PL activity originally present in the cytoplasm. The activity of β-lactamase in culture fluids and in periplasmic and cytoplasmic fractions was determined by measuring the decrease in A_{230} in a 0.5-ml reaction mixture containing 0.1 M potassium phosphate (pH 7.0), 250 mg of penicillin G, and enzyme sample as previously described by Sykes and Matthew (38).

Analysis of PL proteins. PLs produced by *P. fluorescens* CY091 and *E. coli*(pROTM2) were prepared and analyzed by the methods previously described (22, 32). Osmotic shock fluids from *E. coli*(pROTM2) and *E. coli*(pUC18) were concentrated by ultrafiltration (PM10 membrane; Amicon Corp., Danvers, Mass.). Final concentrations of samples were adjusted to 0.2 to 0.3 U of PL activity μl^{-1} (0.4 to 0.6 μg of proteins μl^{-1}). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was conducted according to the method of Laemmli (20) with 3 to 7 μg of protein per gel lane. Isoelectric focusing (IEF) of the sample was conducted in premade thin-layer polyacrylamide gels (PAG plates, pH 3.5 to 9.5; Pharmacia-LKB Biotechnology, Piscataway, N.J.) as previously described (20, 32). The PL sample (3 to 8 μl), containing 0.5 to 0.8 U of PL activity, was applied directly onto the gel. After electrophoresis, gels were either stained with Coomassie blue to detect proteins or subjected to agarose overlay techniques for detection of PL activity (22, 32). To remove SDS present in polyacrylamide gels, gels were rinsed in 750 ml of 50 mM Tris · HCl (pH 8.0) with gentle shaking for 2 h (19). Washed gels were then pressed against pectate-agarose overlays (22) and incubated overnight at 28°C. Bands corresponding to PLs were visualized by submerging gel overlays in 1% mixed alkyltrimethyl ammonium bromide and appeared as clear areas in an opaque background.

Preparation of DNA probes and Southern blot analysis. The nucleotide sequence of a *pel*-containing 1.7-kb (*Xho*I-*Sal*I) fragment described in this report has recently been determined (9a). The 0.7-kb (*Bgl*II-*Cla*I) internal fragment, which has been shown to encode the *pel* structural gene, was isolated from pROTM2. This *pel*-specific fragment was modified and labeled by chemical methods (39), using a Chemiprobe detection kit (FMC Corp., Rockland, Maine) according to the manufacturer's instructions. Chromosomal DNA was isolated by the method of Shepard and Polisky (34) and further purified on an Elutip-d minicolumn (Schleicher & Schuell). DNAs were exhaustively digested with *Eco*RI or *Sal*I (3 to 7 U/μg of DNA). Southern blot procedures were performed as previously described (27). Probe DNA was added at a concentration of 0.5 to 1.0 μg of DNA per ml of hybridization solution. For detection of specific homologous bands, blots were washed under highly stringent conditions as suggested by the manufacturer of the detection kit.

TABLE 3. Effect of Ca^{2+} ions and carbon source on pectate lyase production by *P. fluorescens* CY091

Medium[a] containing:	Total activity ($U/10^9$ cells)[b]	%[c]	
		Extracellular	Intracellular
Glucose	0.90	12.2	87.8
Glucose + Ca^{2+}	4.14	96.0	4.0
Glycerol	0.54	21.9	78.1
Glycerol + Ca^{2+}	6.25	90.1	9.9
PGA	ND	ND	ND
PGA + Ca^{2+}	5.43	87.8	12.2

[a] The MS solution contained K_2HPO_4 (0.7%), KH_2PO_4 (0.2%), $MgSO_4 \cdot 7H_2O$ (0.7%), and $(NH_4)_2SO_4$ (0.1%) and was supplemented with glucose (0.2%), glycerol (0.2%), or PGA (0.4%). $CaCl_2$ was added at a final concentration of 1 mM.

[b] Each value represents an average of two experiments, one replicate per experiment. One unit of activity is defined as the amount of enzyme which causes an increase of 1.73 absorbance units at 30°C per min under the assay conditions described in the text. ND, not determined. The organism failed to grow with PGA as a sole carbon source in the absence of Ca^{2+}.

[c] Activities in culture supernatants and in sonicated cell extracts were considered as extracellular and intracellular activities, respectively.

RESULTS

Effects of Ca^{2+} on PGA utilization and PL production. *P. fluorescens* CY091 is nutritionally versatile and capable of using a wide variety of carbohydrates, including glucose, glycerol, and PGA, as carbon (C) sources. When glucose, glycerol, or other monosaccharides (25) were included as a C source, the MS solution was sufficient to support the growth of this pseudomonad. However, when PGA was included as the sole C source, the organism was unable to grow in MS medium unless $CaCl_2$ (1 mM) was also added. In MS medium containing glucose or glycerol, the growth rate and yield of strain CY091 was not improved with the addition of $CaCl_2$. Thus, Ca^{2+} is required for the utilization of PGA but not of glucose or glycerol.

Production of PL by strain CY091 grown under various conditions was investigated. Results (Table 3) show that production of PL was not markedly affected by the type of C source but was affected by the presence or absence of Ca^{2+} in the culture media. In the presence of $CaCl_2$, this pseudomonad produced high levels of PL ranging from 4.14 to 6.25 $U/10^9$ cells in media containing glucose, glycerol, or PGA. In the absence of $CaCl_2$, the organism produced much lower levels of PL (0.54 to 0.90 $U/10^9$ cells) in media containing glucose or glycerol. This is equivalent to 8 to 20% of the levels detected in Ca^{2+}-containing media. Moreover, Ca^{2+} affected not only the amount but also the location of PL produced. When the organism was grown in Ca^{2+}-deficient media, over 78% of total PL produced was detected within the cells. However, when it was grown in medium containing $CaCl_2$, over 87% of total PL was excreted into culture fluids. These results (Table 3) reveal for the first time that divalent metal ions such as Ca^{2+} are required for the production and secretion of PL by *P. fluorescens*.

Cloning and characterization of the *pel* gene. A genomic library of *P. fluorescens* CY091 was constructed in *E. coli* HB101 by using pBR322 as a vector. Of approximately 2,000 Ap^r transformants screened for pectolytic activity on SSP medium, two gave positive reactions. One clone, designated pROT1, was shown to carry a 16-kb insert at the *Bam*HI site of pBR322. The second clone, carrying an 18-kb insert, was genetically unstable and gradually lost pectolytic activity after three passages in LA-ampicillin medium.

The primary clone pROT1 carrying a 16-kb insert was restricted with *Eco*RI and subjected to deletion subclonings by standard procedures. Deletion derivatives of pROT1 indicated that a 7.1-kb *Eco*RI fragment was sufficient to confer the pectolytic phenotype. When this fragment was transferred into the *Eco*RI site of PBR325 in either orientation, the resulting plasmids in *E. coli* were all pectolytic to about the same degree (pROT3A [Fig. 1] and pROT2A [not shown]). This result indicates that the 7.1-kb *Eco*RI fragment likely contains a promoter active in *E. coli*. Subfragments of the 7.1-kb *Eco*RI fragment were introduced into either pBR325 or pBR322, to generate pROT3A2, pROTX1, pROTX2, and pROTX3, and the resulting plasmids were introduced into *E. coli* and tested for pectolytic activity. The results, summarized in Fig. 1, indicate that a region spanning the center of the fragment is necessary for pectolytic activity. In an independent set of subclonings from the original pROT1 plasmid, a 4.3-kb *Sal*I fragment with pectolytic activity was identified and subcloned into pBR322 (pROT8B; Fig. 1); this fragment was found, by restriction mapping, to overlap with the previously identified *Eco*RI fragment. A 1.7-kb *Sal*I-*Xho*I subfragment, cloned into pUC18, with the *Xho*I site proximal to the vector *lac* promoter (pROTM2; Fig. 1) has pectolytic activity. Constructs having the subfragment inserted in the reverse orientation were nonpectolytic. These results indicate that transcription of the *pel* gene in pROTM2 is likely dependent on the vector *lac* promoter and initiated from the *Xho*I end of the 1.7-kb fragment. This conclusion has recently been confirmed by nucleotide sequence analysis, which shows that the start codon (ATG) and the stop codon (TAA) of the *pel* gene were located respectively at bases 64 and 1204 downstream from the *Xho*I site (9a). *E. coli* cells carrying pROTM2 or pROT8B produced up to 50 to 100 times more PL than did cells carrying a construct (pROT3A or pROT3A2) that is dependent on the *pel* promoter of *P. fluorescens* for transcription. *E. coli* cells carrying pROTM2 formed pits on SSP medium after incubation at 28°C for 20 h, whereas cells carrying pROT3A often took 2 to 4 days to cause visible depressions under the same culture conditions.

Localization of PL in pectolytic *E. coli* cells. *E. coli* cells carrying pROTM2 were grown in MS medium enriched with yeast extract, Casamino Acids, and various carbohydrates. Production of PL by *E. coli*(pROTM2) was not significantly affected by the type of C source included in the medium, nor was it enhanced by the addition of $CaCl_2$. When $CaCl_2$ was not added, total activities of PL in the range of 2.63 to 3.09 $U/10^{10}$ cells were detected with bacteria grown in media containing glucose, glycerol, or PGA. When $CaCl_2$ was added at the final concentration of 1 mM, total activities in the range of 2.65 to 3.22 $U/10^{10}$ cells were detected. Analysis of enzyme activities in subcellular fractions revealed that 78 to 88% of total PL was accumulated in the periplasmic space and that 5 to 12% of the total PL activity was either released into culture fluids or retained inside the cytoplasmic membrane. In each analysis, β-lactamase activities in subcellular fractions were simultaneously monitored to ensure that spheroplasts were properly prepared. The distribution of β-lactamase activity in each subcellular fraction was as follows: culture fluid, 5 to 8%; periplasm, 80 to 85%; and cytoplasm, 10 to 15%.

Characterization of PL from pectolytic *E. coli* cells. Concentrated periplasmic fluids from *E. coli*(pROTM2) and from *E. coli*(pUC18) were analyzed by SDS-polyacrylamide gel electrophoresis and by ultrathin-layer polyacrylamide gel IEF. A PL protein of 41 kDa was detected in periplasmic fluid of *E. coli*(pROTM2) but not in that of *E. coli*(pUC18)

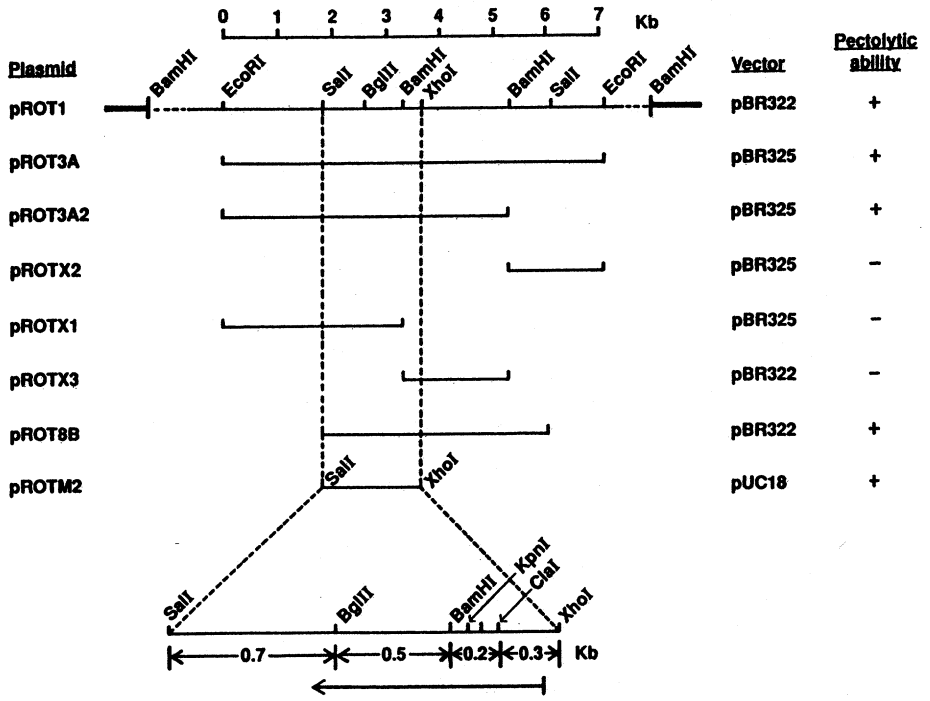

FIG. 1. Restriction map of the cloned region of *P. fluorescens* DNA containing the *pel* gene. The smallest fragment encoding PL was located on a 1.7-kb *Sal*I-*Xho*I region, which contained no restriction site for *Hin*dIII, *Pst*I, *Nhe*I, *Sst*I, *Bgl*I, or *Bcl*I. In pROTM2, the *Xho*I end of the insert is located downstream of the pUC18 *lac* promoter and the *pel* transcription is initiated from the *Xho*I site to the *Sal*I site. In pROT3A, the 7.1-kb *Eco*RI insert likely contains a native *pel* promoter of *P. fluorescens* for self-expression. The positions of the pBR322 Tc^r, the pBR325 Cm^r, and the pUC18 *lac* promoters are at the right end of the cloned fragments depicted. The arrow indicates the position of the *pel* gene and the direction of transcription.

(Fig. 2A). The molecular masses of PLs from *E. coli*(pROTM2) (lane 2) and from *P. fluorescens* CY091 (lanes 3 and 4) are similar or identical. PL activities of these proteins were analyzed and confirmed by using overlay staining techniques (Fig. 2B). Like the PL from *P. fluorescens* CY091, the PL present in shock fluids of *E. coli*(pROTM2) was an alkaline protein, which migrated to the very basic end on IEF gels (Fig. 3A). PL activities of these basic proteins were also confirmed by overlay staining techniques (Fig. 3B). The pI of PL from *E. coli*(pROTM2) was estimated to be 10.0, which is in agreement with the value for its counterpart produced by *P. fluorescens* CY091. These results indicate that the 1.7-kb *Sal*I-*Xho*I insert in pROTM2 encodes an alkaline PL. Unlike *P. fluorescens* CY091, *E. coli* cells carrying pROTM2 were capable of inducing browning and maceration of potato tuber tissue under oxygen stress conditions as described in Materials and Methods. The size of the disease lesion is in the range of 5 to 9 mm in diameter and 1 to 2 mm in depth.

Detection of *pel* homologs in fluorescent pseudomonads. Chromosomal DNAs from 12 strains of bacteria (Table 1) were digested with *Eco*RI or *Sal*I. After electrophoresis in agarose gels and blotting onto nitrocellulose paper, these digests were hybridized with a 0.7-kb *pel*-specific probe. This 0.7-kb (*Bgl*II-*Cla*I) fragment has recently been shown to contain the *pel* structural sequence by nucleotide sequence analysis and transposon mutagenesis (9a). As shown in Fig. 4, *pel* hybrids were detected in *Eco*RI- and *Sal*I-generated genomic digests from seven strains of *P. fluorescens*, one strain of *P. viridiflava*, and two strains of *P. putida*. No *pel* homolog was detected in the genomic digests of *P. fluorescens* 13525 and *E. coli* HB101. In the genomic digest of *P. fluorescens* CY091, a *pel* hybrid was identified in an *Eco*RI fragment larger than 9.4 kb instead of the 7.1-kb fragment as originally expected. The 7.1-kb *Eco*RI insert in pROT3A

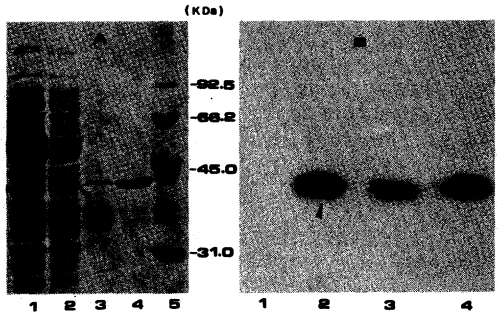

FIG. 2. (A) SDS-polyacrylamide gel electrophoresis of proteins in periplasmic fluids of *E. coli*(pUC18) (lane 1) and *E. coli*(pROTM2) (lane 2) and in culture fluid of *P. fluorescens* CY091 (lane 3). PL purified from culture fluid of CY091 was used as a reference (lane 4). Protein molecular weight standards are phosphorylase, bovine serum albumin, ovabumin, and carbonic anhydrase (lane 5). The arrow indicates the PL protein produced by *E. coli*(pROTM2). (B) Confirmation of PL proteins by agarose-pectate overlay activity stain. A protein (41.0 kDa) having the PL activity was detected in periplasmic fluid of *E. coli*(pROTM2) (lane 2) but not in that of *E. coli*(pUC18) (lane 1). The PL protein of *E. coli*(pROTM2) migrated at a similar rate as its counterpart in culture fluid of *P. fluorescens* CY091 (lane 3) and the purified PL sample (lane 4).

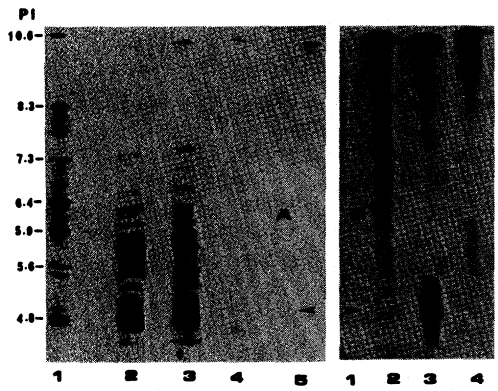

FIG. 3. (A) Ultrathin-layer polyacrylamide gel IEF of proteins present in periplasmic fluids of *E. coli*(pUC18) (lane 2) and *E. coli*(pROTM2) (lane 3). Culture fluid (lane 4) and a purified PL from *P. fluorescens* (lane 5) were included as references. pI markers (horse heart cytochrome *c*, whale sperm myoglobin, equine myoglobin, porcine myoglobin, porcine trifluoroacetyl myoglobin, azurin, and C-phycocyanin) are shown on lane 1. The arrowhead indicates the site where samples were applied. (B) Detection of PL activities of proteins in IEF gels after electrophoresis. Lanes: 1, periplasmic fluid of *E. coli*(pUC18); 2, periplasmic fluid of *E. coli*(pROTM2); 3, culture fluid of *P. fluorescens* CY091; 4, PL purified from culture fluid of *P. fluorescens* CY091.

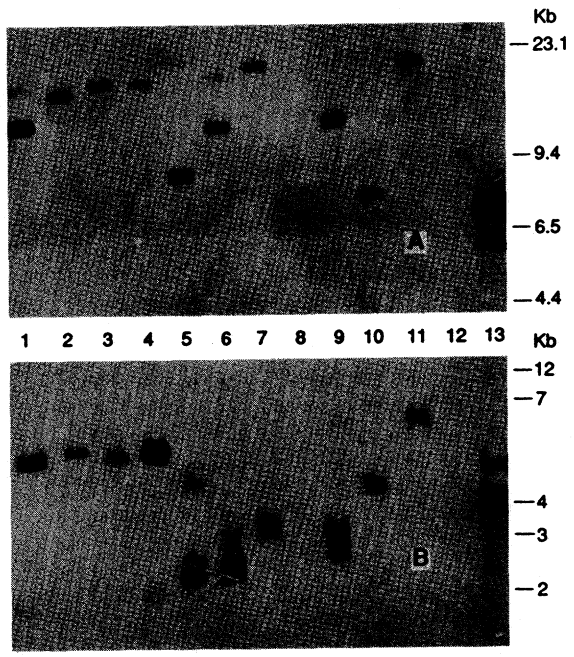

FIG. 4. Detection of *pel*-homologous sequences in various species of fluorescent pseudomonads. Chromosomal DNAs were extracted from *P. fluorescens* strains CY091 (lane 1), 17816 (lane 2), PJ-08-30 (lane 3), SJ-08-2 (lane 4), BC-05-1B (lane 5), LC-04-2B (lane 6), AJ-06-2A (lane 7), and 13525 (lane 8), *P. viridiflava* strain SF312 (lane 9), *P. putida* strains PP22 (lane 10) and AG8 (lane 11), and *E. coli* HB101 (lane 12). Chromosomal DNAs were completely digested with *Eco*RI (A) or *Sal*I (B) and subsequently hybridized with a 0.7-kb *pel*-specific probe. The *Eco*RI-digested pROT3A (lane 13, panel A) and the *Sal*I-digested pROT8B (lane 13, panel B) were included as references.

likely represents multiple *Sau*3A fragments from different locations on the chromosome of strain CY091. In the *Sal*I-generated genomic digest of strain CY091, the *pel* hybrid was located in a fragment close to 4.3 kb. This suggests that the 4.3-kb *Sal*I insert in pROT8B represents an intact chromosomal *pel* region.

The restriction patterns of genomic fragments containing the *pel* genes appear to be varied among the strains of fluorescent pseudomonads. For example, the *pel* hybrids of *P. fluorescens* strains CY091 and LC-04-2B and *P. viridiflava* strain SF312 were located in an *Eco*RI fragment of about the same size (Fig. 4A, lanes 1, 6 and 9). However, the *pel* hybrids in other strains of *P. fluorescens* and *P. putida* were located in a fragment either smaller or larger than that encoded by the *pel* of strain CY091. Despite the difference in the size of DNA fragment, the *pel* homolog was repeatedly detected in the genomic digests prepared from two strains of *P. putida*. Moreover, the presence of a *pel* hybrid in the genomic digest generated by a single restriction enzyme (*Eco*RI or *Sal*I) indicates that the *pel* gene is well conserved in fluorescent pseudomonads and may remain repressed in phenotypically nonpectolytic strains or species such as *P. putida*.

DISCUSSION

The data presented in this report demonstrated that Ca^{2+} plays a critical role in regulation of PL production in *P. fluorescens*. In media containing $CaCl_2$, this pseudomonad constitutively synthesizes and excretes over 87% of PL into culture fluids. In Ca^{2+}-deficient media, the organism produces very low levels of PL and retains the majority of PL within the cells. Genetic and biochemical mechanisms by which Ca^{2+} affects PL production are presently unknown. It appears that Ca^{2+} affects not only the amount of PL synthesized but also the location of PL in subcellular compartments. During the course of this study, attempts to determine the exact location of PL in *P. fluorescens* grown in Ca^{2+}-deficient media were unsuccessful. The failure is mainly due to the lack of appropriate periplasmic protein markers and the lack of reproducible methods for generation of spheroplasts for pseudomonads grown in Ca^{2+}-deficient media. This information will be important for us to understand whether Ca^{2+} is required for translocation of PL from cytosol to periplasm or from periplasm to culture fluids. In *Erwinia chrysanthemi* (5) and *P. viridiflava* (24), the genes (*out*) controlling the export of PL have been identified, and one of them has recently been cloned and characterized (13). The relationship between *out* gene function and Ca^{2+} effect is presently obscure. Whether an interaction between Ca^{2+} and *out* gene products is required for efficient export of PL needs to be further investigated.

Previous studies (2, 16) have shown that bacteria require incorporation of Ca^{2+} into the cell wall to maintain cell wall integrity. In the absence of Ca^{2+}, bacterial cell walls may exist in an unstable form (16) that restricts macromolecule penetration. In other microbial systems, divalent cations have been known to affect enzyme production. For example, Reverchon et al. (31) reported that production of PLa and PLd by *E. coli* clones carrying the *pelA* and *pelD* genes of *E. chrysanthemi* was enhanced by the addition of $CaCl_2$ into culture media. McQueen and Schottel (29) showed that induction of an extracellular esterase was mediated by zinc ions. Kirk et al. (15) demonstrated that production of lignin-ase by *Phanerochaete chrysosporium* was improved with the addition of divalent metal ions (such as Ca^{2+}) into culture media. Although all of these observations further

support the notion that divalent ions play a role in regulation of extracellular enzyme production, none of the foregoing studies included experiments to determine whether the increase in enzyme production is due to the improvement in enzyme-exporting efficiency.

In addition to its role in regulation of PL production, Ca^{2+} is also required for the catalytic action of PL (9, 12) and possibly for transport of PL-generated products into the cells. This study shows that *P. fluorescens* is unable to use galacturonan as a carbon source unless $CaCl_2$ is present in culture media. This finding indicates that initial degradation of pectic substrates present in culture media or in plant cell walls is mediated by Ca^{2+}-dependent PL but not by Ca^{2+}-independent polygalacturonase. Inability to produce polygalacturonases has been previously demonstrated in a number of *P. fluorescens* strains, including strain CY091 (23a, 30). The availability of Ca^{2+} in infected plant tissue therefore becomes a critical factor that determines (i) whether *P. fluorescens* can produce sufficient amounts of tissue-macerating PL; (ii) whether the PL produced is capable of carrying out catalytic action on pectic components of plant cell walls; and (iii) whether *P. fluorescens* can utilize the pectic substances of plant cell walls as a nutritional source. Since all of these events are keys to the development of soft rot, it may be possible to control soft rot caused by *P. fluorescens* by manipulating the availability of free Ca^{2+} in plant infection courts. It should be noted, however, that Ca^{2+} is also important in maintaining the strength of plant cell walls through the formulation of Ca^{2+} bridges among pectic polymers (17). Further research on how to make Ca^{2+} tightly bound to cell walls and make it inaccessible to infecting *P. fluorescens* may lead to the development of new means for control of this postharvest pathogen.

The PL of *P. fluorescens* CY091 has been previously investigated by using IEF electrophoresis and overlay activity staining (22). In addition to the alkaline PL described here, a neutral PL (pI 6.7) band was also observed on the activity-staining gel. The neutral PL can be detected only when a large amount of enzyme sample is applied (22). This suggests that the neutral PL constitutes a minor proportion of total PL produced. Since the gene encoding this neutral PL was not identified in the genomic library constructed during this study, the origin of the neutral PL band on activity-staining gels remains obscure and may represent an artifact derived from the sample trailing effect (22). In DNA hybridization studies (Fig. 4), only one *pel* homolog was detected in the genomic digest of strain CY091. This result indicates that the neutral PL gene, if it does exist, may be located in the same *Eco*RI or *Sal*I fragment encoding the alkaline PL gene. Further analysis of the cloned *pel* fragment with transposon mutagenesis and marker exchange would allow us to determine whether strain CY091 carries a single or multiple PL genes.

With the exception of strain 15325, all of the *P. fluorescens* strains included in this study have been shown to produce PLs with identical IEF profiles (22). It was previously proposed that *pel* genes of *P. fluorescens* were derived from the same ancestor and were well conserved during evolution (22). Results of this study appear to support this hypothesis. A single *pel* homolog has been consistently detected in each of the five *P. fluorescens* strains examined. Since the *pel* homolog is located in different genomic fragments generated by *Eco*RI or *Sal*I, it is possible that the *pel* genes of fluorescent pseudomonads have been subjected to various types of modification or rearrangement (11) during the evolution.

Expression of the pectolytic phenotype in fluorescent pseudomonads is a variable characteristic that can be influenced by a number of culture conditions (3, 10, 43). Certain strains of *P. fluorescens* and *P. viridiflava* have a tendency to lose pectolytic and pathogenic ability during prolonged culture (3, 23a, 24). On the other hand, Zucker and associates (42–44) reported that saprophytic nonpectolytic isolates of *P. fluorescens* can be converted to pathogenic pectolytic forms by serial subculture in media containing pectin or plant tissue extracts. These studies suggest that some strains of fluorescent pseudomonads which exhibit a nonpectolytic phenotype under one set of conditions may become pectolytic under others. Currently, the pectolytic ability of fluorescent pseudomonads is determined by simply assaying their pectolytic reactions on one or two diagnostic pectate media (25). Obviously this practice is inappropriate, since isolates which show negative reactions on these media may become pectolytic under other conditions. The use of a *pel* gene probe in combination with the polymerase chain reaction technology would provide a more accurate means of assessing the pectolytic and soft-rotting ability of potentially pectolytic isolates. I have used this approach to evaluate the pathogenic (or pectolytic) potential of two strains of *P. putida* that were originally isolated and planned to be used for control of plant diseases (23). Both strains exhibit no pectolytic activity on diagnostic media and lack tissue-macerating ability on potato slices, yet they contain *pel*-homologous sequences in their genomes (Fig. 4, lanes 10 and 11). Although it is unclear whether the *pel* homolog detected in *P. putida* encodes a functional or degenerative form of PL, this study provides the first genetic evidence that the *pel* gene may be present in certain strains or species of fluorescent pseudomonads which exhibit nonpectolytic phenotype under conventional testing conditions. Results presented here also support the work of Zucker and associates (42–44), who found that saprophytic strains of *P. fluorescens* can be induced to become pathogenic, and raise a concern about the safety of using *P. fluorescens* and *P. putida* as biocontrol agents.

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